Neural factors that stimulate ecdysteroid synthesis by the larval ring gland of *Drosophila melanogaster*

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Abstract:
The larval ring gland of *Drosophila melanogaster* is the source of ecdysteroids responsible for larval-larval and larval-pupal molting. An extract prepared from the *Drosophila* larval central nervous system, that presumably contains prothoracicotropic hormone, elicits a significant and dose-dependent in vitro increase in ecdysteroid synthesis by ring glands from wandering third instar larvae. The synthesis of all three ecdysteroids previously identified as ring gland products is elevated by more than two-fold in the presence of neural extract. The maximum response occurs within 30 min and can be sustained for at least two hours after a 30 min exposure to neural extract. No non-neural tissue extracts evoke a response and most of the prothoracicotropic activity originates in the ventral ganglion. However, while extract prepared from larval brains elicits only a slight increase in ecdysteroid synthesis, it enhances the activity of a sub-maximal dose of ventral ganglion extract. This suggests that two or more neural factors, at least one from the brain lobes and another from the ventral ganglion, interact to stimulate ecdysteroid synthesis by the larval ring gland.

Abbreviations: CNS central nervous system; HPLC high performance liquid chromatography; PTTH prothoracicotropic hormone; RIA radioimmunoassay

Introduction

In all insects examined thus far, the brain neuropeptide, prothoracicotropic hormone (PTTH), elicits increased ecdysteroid synthesis by the pro-thoracic glands. The subsequent increase in the hemolymph ecdysteroid titer results in the initiation of the molting process (Gilbert et al. 1981). The regulation of this neuroendocrine axis (brain-prothoracic glands) appears to be particularly amenable to genetic analysis by utilizing *Drosophila melanogaster*, but two related limitations have discouraged this line of investigation. First, potentially interesting mutations of the neuroendocrine axis have been identified almost solely on the basis of lower ecdysteroid levels and developmental phenotypes that may merely accompany an unrelated and/or general defect (e.g. Garen et al. 1977; Redfern and Bownes 1983; Holden et al. 1986). The problem is exacerbated by the fact that a true endocrine-specific mutation would probably affect not only endocrine organs but also target tissues (see Hadorn 1961). Second, the PTTH of *Drosophila melanogaster* has not been identified, either structurally or functionally, and therefore no methods existed to evaluate the activity of this component of the steroidogenic axis.

The ability to maintain the *Drosophila* larval ring gland in vitro provides a basis for direct measurement of the ecdysteroid synthesizing capability of the prothoracic gland cells within this structure (Redfern 1983). Further, the ability to stimulate significantly higher levels of ecdysteroid synthesis in larval ring glands incubated in the presence of neural extract suggests the basis for a physiological assay for *Drosophila* PTTH

(Henrich et al. 1987). Similar assays in other insect species have led to insights regarding PTTH release and action (see Gilbert et al. 1981).

The present study examines the physiological parameters associated with the accelerated rate of ecdysteroid synthesis by the ring gland in the presence of *Drosophila* neural extract. The data suggest that one or more neural factors elicit a dose-dependent stimulation of ecdysteroid synthesis by the ring gland.

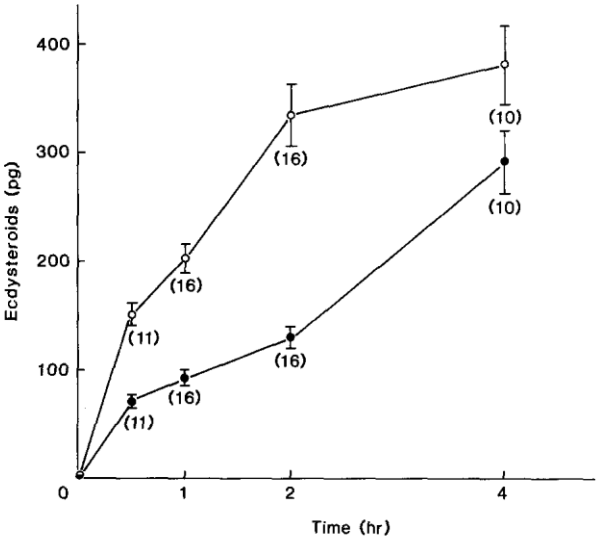

Fig. 1. Time course of neural extract stimulation of ecdysteroid synthesis by *Drosophila* ring glands in vitro. Incubation was at 25 °C for the times indicated. Filled circles (●) denote the mean ecdysteroid levels of incubation medium containing ring glands in Grace's medium alone (control). Open circles (○) denote the mean ecdysteroid levels of incubation medium containing ring glands in Grace's medium with neural extract (experimental). Numbers in parentheses denote sample size for each point and bars indicate SEM

Materials and methods

Animals. A wild-type Canton-S strain of *Drosophila melanogaster* was reared under a photoperiodic regime (16L: 8D) at 25 ± 1 °C because this environment results in enhanced coordination of developmental rate among larvae (Roberts et al. 1987). Special consideration was given to the preparation of the standard agar food medium used to rear larvae since diet can affect the qualitative nature of ecdysteroids synthesized by the ring gland (Redfern 1986). Early wandering larvae were selected for dissection and the ring glands prepared for in vitro incubation by previously described methods (Henrich et al. 1987). In vitro incubations were carried out at 25 °C.

Preparation of extracts. All neural extracts were prepared as described previously (Henrich et al. 1987) and consisted of homogenization in Grace's medium, boiling for 1 min, and using the supernatant after centrifugation at 5000 g (Bollenbacher et al. 1979). In addition, extracts from salivary glands, eyeantennal imaginal discs, and fat body from wandering third instar larvae were prepared in a similar manner. To insure that these extracts were equivalent, the protein oontent of each extract was determined (Lowry et al. 1951). At 8 tissue equivalents per 10 the variation in protein content among the extracts was less than 20%.

Experimental protocols. All data points were based upon independently drawn samples (i.e. individual ring glands measured once), except for those ring glands measured for activity after preincubation (e.g. Fig. 2). For the latter experiments, ring glands were incubated with neural extract for 0.5 h and 13.5 gl of the total 15 gl incubation medium were drawn off and replaced with fresh Grace's medium. This replacement medium was immediately removed and added to the preincubation medium for the measurement of ecdysteroids.

Finally, fresh Grace's medium was added and the ring glands allowed to incubate for 1 or 2 h. Control groups that utilized neural extract or Grace's medium exclusively were tested simultaneously during both preincubation and incubation.

For subsequent reverse phase high performance liquid chromatography (RP-HPLC) analysis, individual ring glands were incubated for 2 h at 25 °C in either Grace's medium alone or Grace's medium containing neural extract. The media from individual samples were pooled for subsequent separation of individual ecdysteroids.

In all studies, the amount of ecdysteroids secreted by individual ring glands was quantified by radioimmunoassay (RIA) as described previously (Warren et al. 1984; Warren and Gilbert 1986). For these experiments, all eodysteroid levels are expressed as ecdysone equivalents and the reported rates of ecdysteroid synthesis were adjusted for the quantity of ecdysteroids detected in the relevant tissue extract. The latter levels never exceeded 30 pg per 15 gl of incubation medium.

High performance liquid chromatography (HPLC) and RIA. The ring gland culture medium was subjected to ion suppression RP-HPLC as described by Pak and Gilbert (1987). The ecdysteroids were fractionated on a C-18 column (Waters, 3.9 mm x 15 cm, Resolve 5 μm spherical packing) and eluted in a gradient mode with an acetonitrile/aqueous buffer (20 m*M* Tris/perchlorate, pH 7.5). The eluted fractions were divided into aliquots and evaporated prior to RIA. One aliquot from each fraction was tested with an antibody (H-22) that reaots with side chain modified ecdysteroids. Each fraction was also analyzed with the H-2 antibody that recognizes ecdysteroids with a modified A-ring (see Warren and Gilbert 1986). Therefore, the RIA not only determined the quantity of ecdysteroids present, but also allowed for tentative characterization of those ecdysteroids for which standards were available.

Results

Time course of response of ring glands to neural extract

The utility of an assay for prothoracicotropic activity of *Drosophila* tissue extracts depends in part on the selection of an incubation time that generates maximal differences between ring glands incubated in Grace's medium alone (basal) and those incubated in Grace's medium containing neural extract (stimulated). The kinetics of response to neural extract can then be compared to that observed in other species for which the action of PTTH upon the prothoracic glands has been examined.

Initial experiments confirmed that ring glands from early wandering larvae synthesize ecdysteroids at a linear rate for as long as 4 h when placed in vitro (Redfern 1984). Nevertheless, incubation with neural extract for 4 h caused only a small increase in the rate of synthesis (Fig. 1). With shorter incubation times (0.5, 1, and 2 h), the ratio of synthesized ecdysteroids between stimulated and control glands varied between 2 and 3, with a maximum difference of 200 pg at 2 h. The plateau in the rate of synthesis seen in stimulated ring glands after 2 h in vitro may have resulted from the depletion of endogenous substrate for ecdysteroid synthesis. Measurement of ecdysteroid levels in the ring glands, as opposed to levels in the incubation medium, after each of these incubation times revealed that neither the experimental nor the control glands contain measurable (i.e. > 15 pg/gland) RIA positive material.

The PTTH of *Manduca sexta* not only activates the prothoracic glands within a few minutes but also causes a prolonged acceleration of ecdysteroid synthesis, even if the prothoracic gland is incubated in the absence of PTTH after a short exposure to the peptide (Bollenbacher et al. 1983). This observation supports the concept that PTTH may be released in a short pulse that subsequently leads to a long-term change in the level of ecdysteroid synthesis. In *Drosophila*, ring glands exposed to neural extract for 30 min and then incubated in Grace's medium alone synthesized about the same amount of ecdysteroids in the next two hours as ring glands incubated continuously in neural extract (Fig. 2). Thus, the *Drosophila* PTTH (neural extract) appears to elicit relatively long-term changes in the ability of the prothoracic gland cells to produce ecdysteroids.

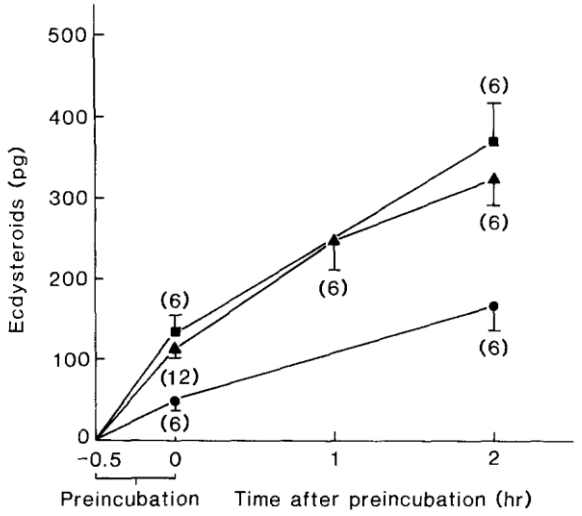

Fig. 2. Effect of preincubation on ecdysteroid synthesis by the ring glands. All ring glands were dissected from early wandering third instar larvae and incubated in 15 µl of medium at 25 °C. Circles (●) denote ring glands preincubated for 30 min and then incubated for 2 h in Grace's medium alone. Squares (■) denote ring glands preincubated for 30 min in Grace's medium containing neural extract and then incubated for 2 h in Grace's medium containing neural extract (8 brain-ventral ganglia/ 10 µl). Triangles (▲) denote ring glands preincubated in Grace's medium containing neural extract for 30 min and then incubated in Grace's medium alone for an additional 2 h. Sample sizes are denoted in parentheses and bars indicate SEM

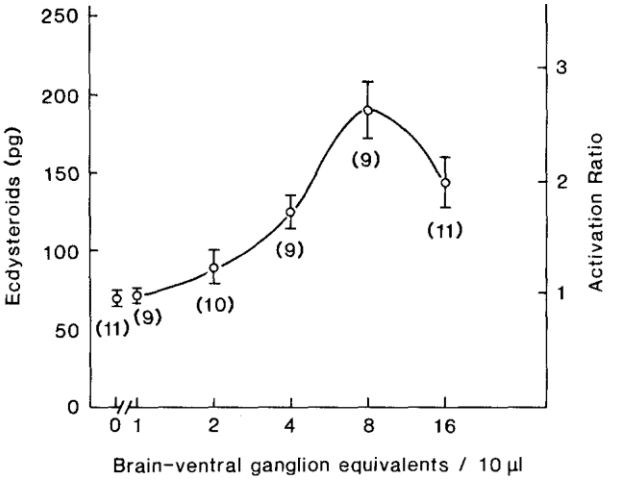

Fig. 3. Dose response of ecdysteroid synthesis by ring glands in the presence of neural extract. Ring glands were incubated at 25 °C for 1 h in Grace's medium containing dilutions of a neural extract. Sample sizes are denoted in parentheses and bars indicate SEM. The activation ratio designates multiples of the mean level of ecdysteroids produced in 1 h by ring glands incubated in Grace's medium alone

Dose-dependence and tissue specificity of ring gland response

If the accelerated rates of synthesis observed in vitro in the presence of neural extract truly reflect a physiological phenomenon, then the level of ecdysteroid synthesis should increase to a maximum level as the concentration of neural extract is increased. The concentrations of extract chosen for this study were based on the doses of PTTH that stimulate the prothoracic glands of *Manduca* (Bollenbacher et al. 1979), after adjustment for the greater hemolymph and brain volumes in *Manduca*. Serial dilutions of neural extract evoke a range of response from ring glands in vitro that approximates a sigmoid curve (Fig. 3). The inhibition of response seen at the highest dose tested (16 brain-ventral ganglion equivalents) resembles the effect found when *Manduca* prothoracic glands are subjected to a high concentration of PTTH (Bollenbacher et al. 1984).

If the assay reflects the activity of one or more neural-specific substances (i.e. PTTH), then similarly prepared extracts from other larval tissues should not generate an increase in ecdysteroid synthesis. Furthermore, if the analogy between *Manduca* and *Drosophila* holds, then extract prepared from brain lobes should contain most of the prothoracicotropic activity (Agui et al. 1979). It should be noted that the ventral ganglia of *Drosophila* are fused into a single structure that corresponds to the entire ventral nerve cord of *Manduca*.

Extracts of non-neural tissues showed no prothoracicotropic activity when incubated in Grace's medium with ring glands and most of the activity of the brain-ventral ganglion complex was associated with the ventral ganglion (Table 1) although the increase elicited by brain lobe extract was statistically significant ($P < 0.01$, *t*-test). In this set of experiments, 5 of the 31 (16.1%) individual ring glands incubated with brain extract produced more than 150 pg ecdysteroids in one hour, whereas 16 out of 20 ring glands (80%) exposed to ventral ganglion extract produced at least 150 pg of ecdysteroids. Only 2 of 38 control ring glands (5.3%) exceeded this level of activity. This unexpected observation involving the ventral ganglion was explored further.

Table 1. Specificity of neural extract stimulation in vitro of ecdysteroid synthesis by ring glands from wild-type early wandering third instar larvae of *Drosophila melanogaster*[a]

Tissue source	Sample size	Ecdysteroids (pg ± SEM)	Activation ratio
None (Grace's medium alone)	38	76.00 ± 4.89	1.00
Eye-antennal imaginal discs	11	59.48 ± 6.86	0.78
Salivary glands	6	92.17 ± 13.55	1.21
Fat body	6[b]	79.38 ± 6.78	1.04
Brains-ventral ganglion	17	200.15 ± 10.03[c]	2.63
Brains	31	117.07 ± 8.11[c]	1.54
Ventral ganglia	20	187.26 ± 10.07[c]	2.46

[a] Incubations were at 25 °C for 1 h. All tissue extracts were administered at a concentration of 8 tissue equivalents/10 µl and data have been adjusted for endogenous ecdysteroids in tissue extracts
[b] Doses of fat body extract which contain about four times the protein content of a maximally active ventral ganglion extract evoke a significant increase in ecdysteroid synthesis
[c] Statistically significant deviation from basal level of ecdysteroid synthesis (no tissue present, Grace's medium alone) as determined by Student's *t*-test ($P < 0.01$)

Interaction between brain lobe and ventral ganglion substances

Separate brain and ventral ganglion extracts were tested over a range of doses to establish the maximal level of activity obtainable from each extract. As a control, the separate extracts were combined to produce an extract (brain + ventral ganglion) similar to that tested previously (Fig. 3). Over the range of doses tested, brain lobe extract elicited a small response of questionable biological significance from the ring gland, while the ventral ganglion extract appeared to contain a factor that clearly accelerated ecdysteroid synthesis in a dose-dependent manner (Fig. 4). Nevertheless, the combined brain-ventral ganglion extract was more effective in stimulating ecdysteroid synthesis by the ring glands.

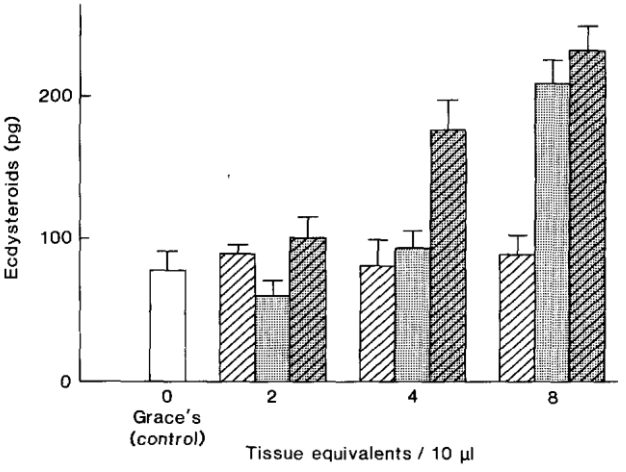

Fig. 4. Interaction of brain and ventral ganglion extract upon ecdysteroid synthesis by ring glands in vitro. Incubation lasted for 1 h at 25 °C. Open bar indicates control ring glands incubated in Grace's medium alone. Hatched bars denote ring glands incubated in Grace's medium containing brain extract. Stippled bars denote ring glands incubated in Grace's medium containing ventral ganglion extract. Stippled-hatched bars denote ring glands incubated in Grace's medium containing a mixture of the brain and ventral ganglion extracts (1:1). For all groups, the data are based upon a sample size ≥ 5. Error bars indicate SEM

In order to examine this phenomenon in further detail, a submaximal dose of ventral ganglion extract (final concentration: 4 ventral ganglia/10 gl) was mixed with extracts of 2, 4 or 8 brain lobe equivalents per 10 id (final concentration) and subjected to the ring gland assay. These experiments revealed that the maximal

response occurs in the presence of ventral ganglion extract only, but that the level of response at submaximal doses increases as the concentration of brain lobe extract increases (Fig. 5).

Changes in individual ecdysteroids in response to neural extract

RIA analysis of the medium after ring gland incubation and RP-HPLC fractionation revealed the presence of 3 ecdysteroids: ecdysone, an unidentified low polarity ecdysteroid, and a third peak tentatively identified as 20-deoxymakisterone A based on its similarity to the ecdysteroids identified by Redfern (1984) and to its differential interactions with the H-22 and H-2 antisera. (A 20-deoxymakisterone A standard was not available.) If activation by neural extract preferentially increases the level of one or more of these individual ecdysteroids relative to the others, then ring gland activation could be traced to a specific step in the biosynthetic pathway. On the other hand, a general and identical increase in the quantity of each ecdysteroid would suggest that activation involves a general stimulation of ecdysteroid biosynthesis, and possibly the general level of metabolic activity within the cell. In fact, the concentration of all three ecdysteroids in the culture medium increases almost identically in response to neural extract suggesting a generalized mode of action (Fig. 6A; Table 2).

The incubation media were also analyzed with the H-2 antibody that recognizes ecdysteroids secreted by the prothoracic gland cells that have a modified A-ring and are not detected by the H-22 antibody. No ecdysteroids were detected with this antibody that were not detected previously with the H-22 antibody (Fig. 6 B). The reduced immunoreactivity of the H-2 antibody to 20-deoxymakisterone A reflects the reduced sensitivity of this antibody because of the additional methyl group in the side chain of this ecdysteroid.

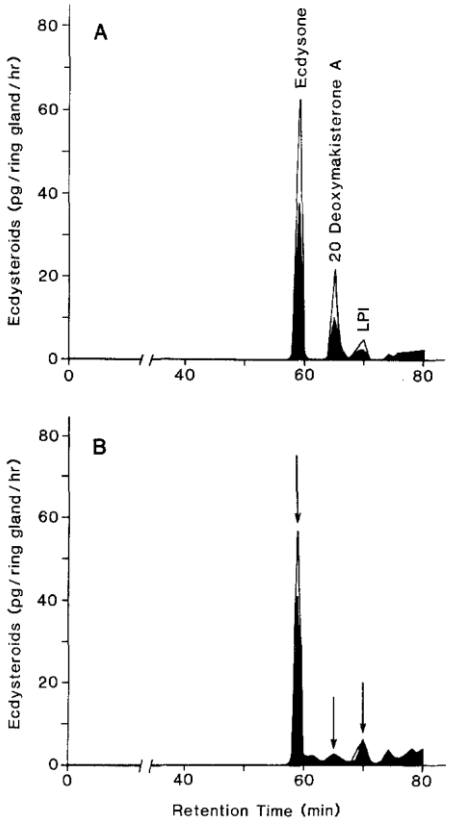

Fig. 6A, B. HPLC-RIA analysis of ecdysteroids present in incubation medium after neural extract stimulation of ring glands in vitro. Aliquots were drawn from one minute elution fractions. **A** Profile after RIA with H-22 antibody. **B** Profile after RIA with H-2 antibody. Shaded portions indicate RIA activity of medium from ring glands incubated in Grace's medium alone at 25 °C for two hours ($N=15$). Unshaded portions indicate RIA activity of medium from ring glands incubated in Grace's medium with neural extract ($N=15$)

Discussion

Extracts prepared from the *Drosophila* larval CNS elicit a dose-dependent and identical increase in the rates of synthesis of the three ecdysteroids known to be produced by the larval ring gland. As with *Manduca* prothoracic glands, the neural extract evokes a maximal stimulation within minutes. Even an exposure as short as 30 min led to a prolonged acceleration of ecdysteroid synthesis over the next two hours.

Collectively, these data suggest that one or more neural factors increase the activity of the ecdysteroid biosynthetic machinery within the pro-thoracic gland cells of the ring gland. This activa- tion persists after removal of the stimulus and may involve changes in translational and/or transcriptional activity within the target cell. If the analogy with *Manduca* holds, then this activation involves a cyclic-nucleotide-protein kinase cascade (Smith et al. 1986). The rapid acceleration of synthesis induced by neural extract and the subsequent drop after two hours corresponds to the rates and patterns of synthesis seen previously in ring glands incubated in vitro after dissection from late wandering larvae (Redfern 1983). This similarity suggests that analogous stimulatory events occur in situ during the wandering larval stage.

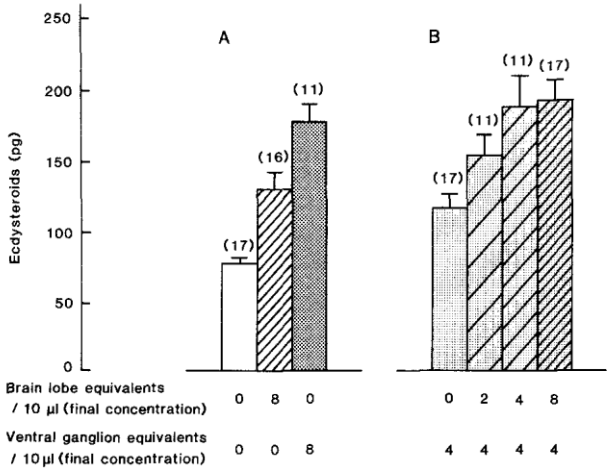

Fig. 5A, B. Ecdysteroids produced by ring glands incubated in Grace's medium containing varying doses of brain and ventral ganglion extract. **A** Designates ring glands incubated in Grace's medium or a maximal dose of brain or ventral ganglion extract for 1 h at 25 °C. **B** Designates ring glands incubated with 4 ventral ganglion equivalents/10 µl (final concentration) and varying doses of brain extract. Bars denote SEM and numbers in parentheses indicate sample sizes

Table 2. Changes in the amount of individual ecdysteroids due to neural extract stimulation of ring glands in vitro

Ecdysteroid	Basal	Stimulated	Activation ratio
	(pg/ring gland/h)		
Ecdysone	38.7	85.6	2.21
20-Deoxymakisterone A	13.5	31.5	2.33
LPI	4.2	10.1	2.40
Total	56.4	127.2	2.25

Ecdysteroids obtained from Grace's medium or medium containing neural extract pooled after 2 h incubation at 25 °C of ring glands from wild-type early wandering third instar larvae. RIA measurements were made with H22 antibody on fractions eluted by reverse phase HPLC and are expressed as ecdysone equivalents. Activation ratio refers to the quantity of ecdysteroid(s) from stimulated ring glands divided by the amount of ecdysteroid(s) from control ring glands

While the active neural factor(s) is heat stable and may be a peptide, the localization of activity in the *Drosophila* ventral ganglion and the enhancing action of one or more brain factors indicates that the *Drosophila* neuroendocrine axis may be organized differently than that of the Lepidoptera, in which the brain is the sole source of a big and little PTTH (see Bollenbacher et al. 1979, 1984). The stimulation of ecdysteroid synthesis observed here is related temporally to an event that does not occur in Lepidoptera, i.e. pupariation. The possibility that several peptide factors control pupariation and molting in Diptera is not a new concept (see Fraenkel et al. 1972), but obviously deserves further investigation.

The ring gland contains the corpus allatum and corpus cardiacum as well as the prothoracic gland. We have not ruled out the possibility that the increase in ecdysteroid synthesis noted here may involve the indirect activation of the prothoracic glands via these other structures. It is known from studies on Lepidoptera that a neural peptide fac tor, allatotropin, regulates aspects of juvenile hormone synthesis (e.g. Granger et al. 1984) and that juvenile hormone can affect ecdysone synthesis by the prothoracic glands of *Manduca* (Rountree et al. 1987). Thus, factors other than PTTH could be acting indirectly to modify ecdysteroid synthesis by the ring gland.

Multiple PTTH moieties exist in other insects (Gilbert et al. 1981), although nothing has been reported concerning their possible interactions. Complementary effects by two or more factors on the ability of a target tissue to perform its function is not unprecedented, as exemplified by the interactive effects of insulin-like growth factors and insulin upon mammalian target tissues (Froesch et al. 1985). The experiments reported here on the interaction between brain and ventral ganglion extracts on the *Drosophila* larval ring gland do not distinguish between additive or synergistic effects but this question is being actively investigated in this laboratory.

The highly compressed duration of the final instar of *Drosophila* (2 days) compared to that of insects such as *Manduca* (9-10 days), at a time when several critical endocrine events occur in rapid succession, will hamper attempts to resolve the issue of steroidogenic regulation using an exclusively physiological approach. Nevertheless, the consistent and predictable changes in ecdysteroid synthesis brought about by exposure of ring glands to neural extract should allow further characterization of the larval neuroendocrine axis. Specifically, the in vitro assay described here provides the capability to identify the foci of genetic lesions within the neuroendocrine axis (Henrich et al. 1987) and should aid in the identification of the critical components of the axis.

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